

11 β -Hydroxylase and aldosterone synthase expression in fetal rat hippocampal neurons

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Abstract

The central nervous system produces many of the enzymes responsible for corticosteroid synthesis. A model system to study the regulation of this local system would be valuable. Previously, we have shown that primary cultures of hippocampal neurons isolated from the fetal rat can perform the biochemical reactions associated with the enzymes 11 β -hydroxylase and aldosterone synthase. Here, we demonstrate directly that these enzymes are present within primary cultures of fetal rat hippocampal neurons.

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Introduction

A considerable body of evidence has now accumulated to suggest that corticosteroid synthesis occurs in tissues other than the adrenal cortex, most notably the cardiovascular system and the central nervous system (CNS) (Strömstedt & Waterman 1995, Gómez-Sánchez *et al.* 1996, Silvestre *et al.* 1998, Kayes-Wandover & White 2000). The *CYP11B1* gene encodes the 11 β -hydroxylase enzyme while the highly homologous *CYP11B2* gene encodes aldosterone synthase. We have shown that the rat brain transcribes both of these genes and that expression of the resulting corticosteroidogenic enzymes localises to the hippocampus and the cerebellum (MacKenzie *et al.* 2000a). In addition, work by Furukawa *et al.* (1998) has demonstrated that transcription of the genes for the side-chain cleavage enzyme (*CYP11A1*), 3 β -hydroxysteroid dehydrogenase, and the steroidogenic acute regulatory protein (StAR) localises to the same regions of the brain and have an identical distribution within those areas. These earlier studies did not confirm that there was active steroid synthesis as a consequence of this mRNA

expression. However, we have recently shown that incubation of 11-deoxycorticosterone (DOC) with a primary culture of hippocampal neurons isolated from the fetal rat results in the production of corticosterone and aldosterone (MacKenzie *et al.* 2000b). This strongly suggests the presence of the 11 β -hydroxylase and aldosterone synthase enzymes within these cells and, as such, would render them a valuable model for the study of neural corticosteroid production.

The aim of this study was to detect the presence of *CYP11B1* and *CYP11B2* transcripts in primary cultures of fetal hippocampal neurons by RT-PCR and immunocytochemistry, and to demonstrate that this transcription results in immunoreactive 11 β -hydroxylase and aldosterone synthase. We also investigated the transcription of the *CYP11A1*, StAR and adrenodoxin genes within these cells. Adrenodoxin is a vital cofactor required by the cytochrome P450 enzymes in the pathway. The *CYP11A1* gene encodes the side-chain cleavage enzyme which catalyses the initial conversion of cholesterol to pregnenolone, while StAR performs the rate-limiting step in corticosteroid synthesis, presenting cholesterol to the side-chain cleavage

Table 1 Oligonucleotide primer sequences used for PCR amplification of cDNA

Primer		Sequence (5'–3')	Product length (bp)
CYP11B1/2	Sense	AAC TCC GTG GCC TGA GAC G	342
	Antisense	GCT GTG TGG TGG ACT TGA AC	
CYP11A1	Sense	CAA CAT CAC AGA GAT GCT GGC AGG	583
	Antisense	CTC AGG CAT CAG GAT GAG GTT GAA	
StAR	Sense	TAC ATT CAA GCT GTG TGC TG	318
	Antisense	TTA CTT AGC ACT TCA TCT CC	
Adrenodoxin	Sense	GAC TCT CTG CTA GAT GTT GTG ATT	289
	Antisense	ATT CTT GCT CAT GTC AAC AGA CTG TCG	

enzyme (Arakane *et al.* 1998). The transcription of these genes would be a strong indication that these cells are capable of *de novo* corticosteroid synthesis from cholesterol.

Materials and methods

Preparation of primary rat fetal hippocampal neuronal cultures

Cultures were prepared from hippocampi dissected out of embryonic day 18 Wistar rats (Banker & Goslin 1991). Tissue was washed three times in Hanks' balanced salt solution (Life Technologies) containing Hepes buffer, pH 7.4, and then trypsinised (1 mg/ml) for 10 min at 37 °C and dissociated by trituration through a 25 gauge sterile needle. Cells were seeded at a density of 2×10^6 cells per six-well poly-L-lysine-coated tissue culture plate (Fred Baker Scientific, Runcorn, Cheshire, UK) or 4×10^5 cells per eight-well culture slide (Fred Baker Scientific) in neurobasal medium containing B27 growth supplement and 0.5 mM L-glutamine and grown in a humidified incubator at 37 °C/5% CO₂. The next day, cytosine arabinoside (5 μ M) (Sigma, Poole, Dorset, UK) was added to the medium to inhibit glial proliferation. Cells were left to grow for 7 days before experiments were performed. Cultures contained less than 5% glia as determined by immunocytochemical staining with glial fibrillary-associated protein antibody.

RNA isolation

Total RNA was extracted from cells grown on six-well plates (see above) or from adult female

Wistar–Kyoto rat tissue according to the RNazol B reagent protocol (Biogenesis Ltd, Poole, Dorset, UK). The resulting RNA was DNase-treated using RQ1 DNase (Promega). The quality and concentration of the RNA was confirmed by spectrophotometry and electrophoresis on ethidium bromide-stained agarose gels.

RT-PCR and Southern blotting

Oligonucleotide primers (Oswel, Southampton, Hampshire, UK) homologous to parts of the *CYP11B1/B2*, *CYP11A1*, StAR and adrenodoxin genes were produced (Table 1). RT-PCR amplification was performed using the GeneAmp RNA PCR kit (Perkin Elmer, Warrington, Cheshire, UK) and oligo-dT primers. cDNA was transcribed according to the standard kit protocol. Amplification of *CYP11A1* and adrenodoxin followed the method of Strömstedt & Waterman (1995). Reactions had an initial 10 min denaturation step at 94 °C followed by 35 cycles of 2 min denaturation at 94 °C, 1 min annealing at 55 °C (except for *CYP11B1/2* where it was 60 °C) and 2 min extension at 72 °C. A final 7 min extension step at 72 °C was used in each reaction. Control reactions omitted reverse transcriptase or substituted water for RNA. Adrenodoxin RT-PCR products were visualised on ethidium bromide-stained 2% agarose gels under u.v. light. All other RT-PCR products were visualised by Southern blotting to Hybond-N+ membrane (Amersham Pharmacia Biotech). Products were detected using ³²P-labelled oligonucleotide probes (Oswel). The *CYP11B1*-specific oligonucleotide was 5'-TAA

ACA TTC AGT CCA ATA-3' (bases 556–574, sense); the *CYP11B2*-specific oligonucleotide was 5'-TGG ATG TCC AGC AAA GTC-3' (bases 556–574, sense); the *CYP11A1*-specific oligonucleotide was 5'-GGT GGA GTC TCA GTG TCT CCT TGA TGC TGG CTT TGA G-3' (bases 1199–1163, antisense); the StAR-specific oligonucleotide was 5'-GCA TAC TCA ACA ACC AGG-3' (bases 319–336, sense).

Monoclonal antibody preparation

Synthetic peptides corresponding to hydrophilic areas of aldosterone synthase and 11 β -hydroxylase exhibiting minimal homology were prepared by Research Genetics Inc. (Huntsville, AL, USA). The aldosterone synthase monoclonal antibody was generated by immunising CD-1 mice, as described by Tam (1988), with the multiple antigenic peptide MAP-KVRQNARGSLTMDVQQ corresponding to residues 175–190. The 11 β -hydroxylase monoclonal antibody was generated in Swiss-Webster mice immunised with the peptide KNVYRELAEGRQQS, corresponding to residues 272–285, conjugated to chicken serum albumin. The spleens of the mice with the highest titre were fused to an SP-2 myeloma cell line and the clones selected for their ability to bind immobilised sonicated zona glomerulosa mitochondria from rats on a low sodium diet. Both antibodies were of the IgG1 subclass. The specificity of these antibodies for their respective target enzymes has previously been established by immunostaining of rat adrenal tissue (MacKenzie *et al.* 2000a).

Immunostaining of fetal rat hippocampal neurons

Cells grown on eight-well culture slides (see above) were fixed for immunostaining by immersion in ice-cold acetone for 10 min. Cells were rehydrated through graded ethanol, then rinsed in deionised water and Tris-buffered saline. Subsequent steps were performed using the Dako Catalysed Signal Amplification (CSA) System Peroxidase Kit (Dako Ltd, Cambridge, Cambridgeshire, UK). The cells were incubated with the primary antibody for 1 h at 4 °C. Immunostaining was developed with 3,3'-diaminobenzidine tetrahydrochloride (Dako). Cells were then counterstained with filtered modified Harris haematoxylin solution (Sigma-

Aldrich Co. Ltd, Poole, Dorset, UK) for 5 min, dehydrated and mounted in DPX (BDH Laboratory Supplies, Lutterworth, Leics, UK). Control sections were incubated with mouse IgG1 negative control culture supernatant (Dako). In addition, the specificity of the monoclonal antibodies was confirmed by incubating them with an excess of their respective antigenic polypeptides (Alta Bioscience, Birmingham, UK), thus quenching any antibody binding.

Results

RT-PCR

CYP11B1, *CYP11B2*, *CYP11A1* and StAR transcripts were visible in adrenal gland, brain and fetal hippocampal neuron samples after Southern blotting (Fig. 1). Although semi-quantitative, it was apparent that each transcript was more abundant in adrenal tissue than in brain or fetal hippocampal neurons. Adrenodoxin transcripts were sufficiently abundant in adrenal gland, brain and fetal hippocampal neuron samples to be visible on an ethidium bromide-stained agarose gel under u.v. light. All control reactions were negative.

Immunostaining

Cells stained positively for 11 β -hydroxylase (Fig. 2a) and aldosterone synthase (Fig. 2b). Staining for both enzymes was apparent in the dendrites. Cells incubated with mouse IgG1 as a negative control were unstained (Fig. 2c). Positive immunostaining was quenched by prior incubation of each antibody with an excess of its immunogenic peptide (data not shown).

Discussion

The *CYP11B1* and *CYP11B2* genes are transcribed within primary cultures of fetal rat hippocampal neurons and are subsequently translated to form immunoreactive 11 β -hydroxylase and aldosterone synthase polypeptides. This complements our earlier finding that incubation of these cells with the enzymes' common substrate, DOC, results in the production of their respective products, corticosterone and aldosterone.

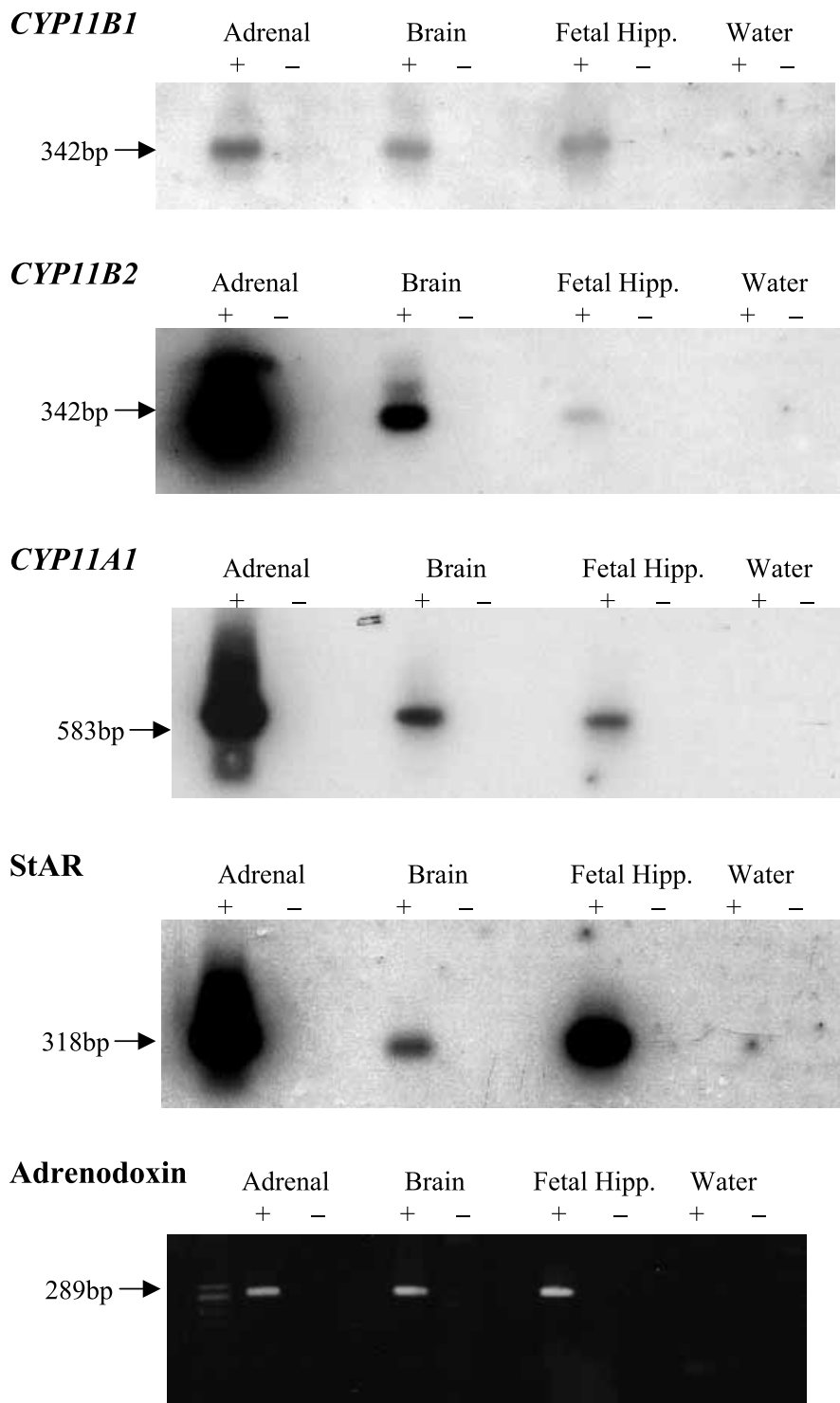


Figure 1 RT-PCR/Southern blotting results, in the presence (+) and absence (-) of reverse transcriptase. Hipp, hippocampus.

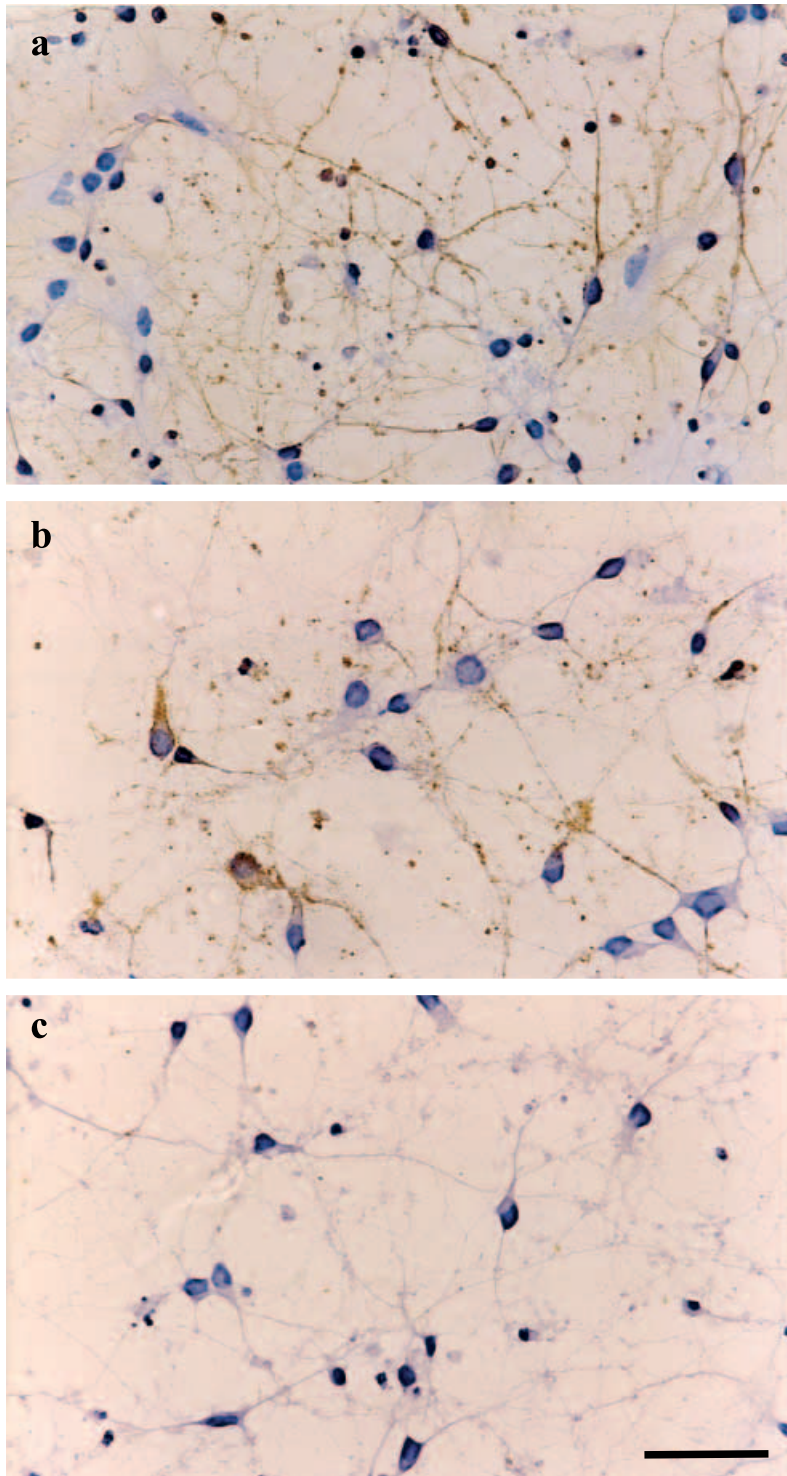


Figure 2 Immunostaining for steroidogenic enzymes in primary cultures of fetal rat hippocampal neurons. Staining for 11 β -hydroxylase (a) and aldosterone synthase (b). (c) Mouse IgG1 negative control. Scale bar=50 μ m.

We have also shown that the genes for the side-chain cleavage enzyme (*CYP11A1*), adrenodoxin and StAR are transcribed in primary cultures of fetal rat hippocampal neurons. The transcription of these genes suggests that primary cultures of fetal rat hippocampal neurons could be capable of *de novo* production of corticosteroids from cholesterol, as well as using circulating DOC as substrate.

We have previously shown that 11 β -hydroxylase and aldosterone synthase are expressed in the adult rat hippocampus and cerebellum (MacKenzie *et al.* 2000a). Other components of the glucocorticoid and mineralocorticoid pathway localise to the same regions of the CNS (Furukawa *et al.* 1998). Moreover, their distributions correlate closely with that of the mineralocorticoid receptor (MR) in the brain (Agarwal *et al.* 1993). It is also apparent from these studies, and from others (Ukena *et al.* 1998, 1999), that all of these genes are expressed within the Purkinje neurons of the cerebellum, as the size of this cell makes expression relatively easy to detect. However, the present study is the first to show that hippocampal neurons have a similar capability. Therefore, we now know that neurons in both the hippocampus and cerebellum express the majority of genes required for both corticosterone and aldosterone synthesis.

It is not clear what the relative contributions of neuronal and glial cells might be to total CNS corticosteroid production. Cultures of glial cells are known to express the side-chain cleavage enzyme and 3 β -hydroxysteroid dehydrogenase but 11 β -hydroxylase could not be detected by RT-PCR (Jung-Testas *et al.* 1989, Mellon & Deschepper 1993). *CYP11B2* transcripts could not be detected in these cells by RNase protection assay, although this technique is less sensitive than RT-PCR. It remains possible that, unlike those enzymes acting earlier in the pathway, CNS expression of *CYP11B1* and *CYP11B2* does not take place in glia but rather in neurons. 11 β -Hydroxysteroid dehydrogenase type 2 is not expressed in the adult rat hippocampus (Robson *et al.* 1998) so active glucocorticoid is free to bind MR within this region. This means that any aldosterone produced as a result of hippocampal aldosterone synthase expression will have to compete with glucocorticoid for MR. The proportion of bound MR will, therefore, be determined by the combined levels of corticosterone and aldosterone, rather than of aldosterone alone.

There is little information on the regulation of expression of these genes in neurons, although it is possible that this differs from that described in adrenal cortex. Our own preliminary studies show that dietary sodium restriction can significantly upregulate *CYP11B2* transcript levels in the rat hippocampus and cerebellum (Ye *et al.* 2002). Therefore, stimuli are capable of inducing predictable physiological responses from this system. The role of local corticosteroid production – as distinct from hormone availability from the systemic circulation or reactivation of dehydrocorticosterone by 11 β -hydroxysteroid dehydrogenase type 1 – remains to be determined. Although the absolute level of expression in the CNS is low, it is possible that high concentrations of steroids acting in the autocrine or paracrine mode are available as a result of neuronal synthesis. Low levels of corticosteroid in the CNS can be capable of significant physiological effects. Doses of aldosterone which have no effect when administered i.v. are capable of raising systemic blood pressure when given through the i.c.v. route (Gómez-Sánchez 1986).

The adrenal corticosteroids can, under different circumstances, have either protective or destructive effects on nerve cells (Sapolsky 1996, McEwen 1999). Obviously, local production of corticosteroids by these very cells could have significant implications for certain neurodegenerative processes. Likewise, further research is required into the effects of corticosteroids in the brain on memory and learning (Schumacher *et al.* 1997, Douma *et al.* 1998), neuronal excitability (Joels 1997) and blood pressure homeostasis (Gómez-Sánchez 1997). The great complexity of the brain, together with the highly specific regional distribution of the corticosteroidogenic machinery throughout the CNS, makes direct study difficult. Models of neuronal corticosteroid production are urgently required for further study. The primary culture of fetal rat hippocampal neurons will be a valuable aid in examining the regulation of local CNS corticosteroid synthesis.

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